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(71) Applicant: ORTHO-MCNEIL PHARMACEUTICAL, INC. [US/US]; US Route 202, Raritan, NJ 08869 (US).

(72) Inventors: DUBIN, Adrienne; 4303 Bromfield Avenue, San Diego, CA 92122 (US). ERLANDER, Mark; 442 Hillcrest Drive, Encinitas, CA 92024 (US). ROGERS, Kathryn, E.; 4036 Redwing Lane, Audubon, PA 19403 (US). HUVAR, Arne; 5560 Shasta Lane #67, La Mesa, CA 91942 (US). HUVAR, Rene; 8732 Crossway Court #35, Santee, CA 92071 (US).

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(54) Title: DNA ENCODING THE HUMAN VANILLOID RECEPTOR VR1

(57) Abstract

DNA encoding human VR1 receptor has been cloned and characterized. The recombinant protein is capable of forming biologically active protein. The cDNA's have been expressed in recombinant host cells that produce active recombinant protein. The recombinant protein is also purified from the recombinant host cells. In addition, the recombinant host cells are utilized to establish a method for identifying modulators of the receptor activity, and receptor modulators are identified.

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TITLE OF THE INVENTION

DNA ENCODING THE HUMAN VANILLOID RECEPTOR VR1

BACKGROUND OF THE INVENTION

Noxious chemical, thermal and mechanical stimuli excite peripheral nerve endings of small diameter sensory neurons (nociceptors) in sensory ganglia (eg., dorsal root, nodose and trigeminal ganglia) and initiate signals that are perceived as pain. These neurons are crucial for the detection of harmful or potentially harmful stimuli (heat) and tissue damage (H^+ (local tissue acidosis), and/or stretch) which arise from changes in the extracellular space during inflammatory or ischaemic conditions (Wall and Melzack, 1994).

Capsaicin (8-methyl-N-vanillyl-6-nonenamide), the main pungent ingredient in "hot" capsicum peppers, and its analogs interact at specific membrane recognition sites (vanilloid receptors), expressed almost exclusively by primary sensory neurons involved in nociception and neurogenic inflammation (Bevan and Szolcsanyi, 1990). Capsaicin is a very selective activator of thinly or unmyelinated nociceptive afferents (Szolcsanyi, 1993; Szolcsanyi, 1996). Capsaicin derivatives show structure-function relationships and their effects can be blocked by a selective antagonist capsazepine. The ultra potent tricyclic diterpene resiniferatoxin (RTX; (Szolcsanyi et al., 1991)) binds with nanomolar affinity at the capsaicin binding site and has revealed a very localized distribution of capsaicin receptors to rat somatic and visceral primary sensory neurons (Szallasi et al., 1995). Interestingly, the density of RTX receptor sites in nodose and dorsal root ganglia increased after ligation of the vagal and sciatic nerves (Szallasi et al., 1995).

Electrophysiological studies have shown that vanilloids excite small sensory neurons by activating a plasma membrane channel that is non-selectively permeable to cations (Bevan and Szolcsanyi, 1990; Oh et al., 1996; Wood et al., 1988).

Recently, one receptor for capsaicin (VR1) was cloned from rat (Caterina et al.,

[redacted]

1997) and shown to be a coincidence detector for H^+ (low pH) and heat (Tominaga et al., 1998). VR1 is expressed in small nociceptive neurons of the dorsal root ganglion, consistent with its role in modulating peripheral pain (Tominaga et al., 1998). The vanilloid ("capsaicin") receptor VR1 is activated by capsaicin and RTX, and activation of VR1 is blocked by the antagonists capsazepine (CPZ; (Bevan et al., 1992)) and ruthenium red (RR; (Wood et al., 1988)) (Caterina et al., 1997). VR1 is a ligand-gated non-selective cation channel that shows pronounced outward rectification (Caterina et al., 1997). Recently, rat VR1 and VR2 and a partial cDNA sequence of human sequences were disclosed in the WIPO publication WO 99/09140.

Topical application of vanilloids such as capsaicin (Zostrix 0.025% and Zostrix HP 0.075%) have been used to mitigate neuropathic pain and to treat the intractable pain associated with postherpetic neuralgia, diabetic neuropathy, postmastectomy pain, complex regional pain syndromes and rheumatoid arthritis (Robbins et al., 1998; Rowbotham, 1994; Szallasi and Blumberg, 1996). With prolonged exposure to capsaicin, nociceptor cells become not only insensitive to this agonist but to other noxious stimuli as well (Szolcsanyi, 1993). The mechanism by which capsaicin produces analgesia is not known but likely includes desensitization of nociceptive sensory neurons, and depletion of peptides from peripheral terminals, as well as damage to sensory nerves (Jancso et al., 1977; Rowbotham, 1994). The irritancy of capsaicin severely limits its use, and the discovery of novel compounds that block the acidic and/or thermal activation of capsaicin sensitive receptors is sought. The antagonists RR and CPZ, while exerting antinociceptive effects in a behavioral study (Santos and Calixto, 1997), have not proven to be effective analgesics in man, presumably because they do not antagonize the endogenous modulators of the capsaicin receptor (Kress and Zeilhofer, 1999). However, CPZ blocked H^+-induced currents from rat VR1 expressed in Xenopus oocytes (Tominaga et al., 1998).

Low doses of capsaicin protected the rat gastric mucosa against injury produced by different ulcerogenic agents (Ome et al., 1997). "The gastro protective effect of capsaicin-type agents involves an enhancement of the microcirculation effected through the release of mediator peptides from the sensory nerve terminals with calcitonin gene-related peptide being the most likely candidate implicated. Capsaicin-sensitive fibers are involved in the repair mechanisms of the gastric mucosa. In most studies, capsaicin given into the stomach of rats or cats inhibited gastric acid secretion (Ome et al., 1997)."

SUMMARY OF THE INVENTION

A DNA molecule encoding the human vanilloid receptor (hVR1) has been cloned and characterized. The biological and structural properties of these proteins are disclosed, as is the amino acid and nucleotide sequence. The recombinant protein is useful to identify modulators of the receptor VR1. Modulators identified in the assay disclosed herein are useful as therapeutic agents, which are candidates for the treatment of inflammatory conditions associated with capsaicin receptor activity and for use as analgesics for intractable pain associated with postherpetic neuralgia, diabetic neuropathy, postmastectomy pain, complex regional pain syndromes, arthritis (e.g., rheumatoid and osteoarthritis), as well as ulcers, neurodegenerative diseases, asthma, chronic obstructive pulmonary disease, irritable bowel syndrome, and psoriasis. The recombinant DNA molecules, and portions thereof, are useful for isolating homologues of the DNA molecules, identifying and isolating genomic equivalents of the DNA molecules, and identifying, detecting or isolating mutant forms of the DNA molecules.

BRIEF DESCRIPTION OF THE DRAWING

FIGURE 1 - The nucleotide sequence of coding region of hVR1 is shown (2520 bp).

FIGURE 2 - The nucleotide sequence of hVR1 is shown including 921 bp 5' UT and 1383 bp 3'UT.

FIGURE 3 - The amino acid sequence of hVR1 is shown (839 amino acids).

FIGURE 4- Functional expression of hVR1 in Xenopus oocytes is shown: activation by capsaicin and resiniferatoxin and block of capsaicin response by capsazepine and ruthenium red. (a). Capsaicin (C; 1 μM) is applied at the time indicated by the bar (left panel). Preincubation with 0.6 μM capsazepine (CPZ) for 2 min blocked residual current still present 6 min after the original response (small outward current in beginning of current trace (middle panel)) and completely blocked subsequent application of C (C + CPZ, middle panel). There was partial recovery of the response to C (right panel) after 6 minutes of continuous rinsing. (b.) Ruthenium red (RR, 1 μM) blocked the C response (middle panel) and the effect was partially reversible after washout of the antagonist. Time scale is indicated by the horizontal bar (50 sec). (c.) Voltage ramp-induced currents before (bottom current trace) and after (indicated with a C) capsaicin (1 μM) application. The membrane potential was ramped from -120 to +80 mV over 200 msec. The arrow indicates 0 mV. The current induced by C is much larger at positive voltages (outward currents) than at negative voltages (inward current) indicating a very strong outward rectification. (d.) A similar current is elicited by resiniferatoxin (RTX, 300 nM).

FIGURE 5- Dose response for capsaicin applied to Xenopus oocytes expressing hVR1. The responses to the indicated concentrations of capsaicin were

bath applied to oocytes expressing 2.5 ng hVR1 cRNA. Oocytes were continuously perfused for 6 min between agonist tests. n=4,3,4,2 oocytes for 0.1, 0.3, 1 and 3 μM agonist, respectively.

FIGURE 6- The oocyte was challenged with 0.6 μM Capsaicin and the maximum response at +80 mV was determined using a voltage ramp protocol. After washing out the capsaicin (6 min), the oocyte was perfused with the indicated concentration of CPZ for 2 min and subsequently tested for its response to 0.6 μM capsaicin in the continued presence of the antagonist. (n= 3 for all values shown.)

FIGURE 7- Functional expression of hVR1 in Xenopus oocytes is shown: activation by low pH (pH 5.5). A voltage ramp protocol was applied to a VR1 expressing oocyte (-120 to +80 mV over 200 ms) and the whole cell currents elicited are shown before (lower current trace in each a-c) and after (current trace indicated with a solid circle) pH5.5 application. Initially oocytes were bathed in ND96 with 100 μM Ca2+, pH 8. Experiment was performed at room temperature (20 deg C). Arrow indicates 0 mV. Low pH activates an outwardly rectifying current (a) that is blocked by CPZ) (b). The effect of CPZ is reversible (c). In this example inward currents are very small, presumably due to the low levels of extracellular Ca^{2+}. Whole cell currents recorded in the presence of low pH are indicated by the solid circles.

FIGURE 8- Functional expression of hVR1 in a mammalian cell line is shown: HEK293 cells were transiently transfected with hVR1 (a) or vector alone (pcDNA3.1-zeo) (b) and 4 days later were tested for their response to vanilloid agonists and antagonists. Ca^{2+} influx was measured using the Ca^{2+} sensitive dye Fluo-4 on a FLIPR system. 1: Cells were challenged with 1 μM capsaicin during the

time indicated by the open bar (duration: about 1.5 min). 2: Cells were preincubated in 100 nM CPZ (solid bar) for about 1 min and then challenged with 1 μM Capsaicin (open bar) in the presence of 100 nM CPZ. 3: Cells were preincubated in 1 μM CPZ (solid bar) for about 1 min and then challenged with 1 μM Capsaicin in the continued presence of 1 μM CPZ. 4: Cells were challenged with 100 nM RTX (grey bar). 5: Cells were preincubated with 1 μM CPZ (solid bar) and then challenged with 100 nM RTX (grey bar) in the continued presence of CPZ. Responses were measured in 15 mM Ca^{2+} buffer. Cells were confluent the day of recording.

Figure 9 A and B. HEK293 cells stably expressing hVR1 were tested for responsiveness to capsaicin and sensitivity to ruthenium red and capsazepine. The increase in intracellular Ca2+ evoked by 15 nM Capsaicin was blocked in a dose dependent manner by ruthenium red (A, B) and capsazepine (C). The parent cell line did not respond to capsaicin (top 2 rows).

Figure 10 HEK293 cells stably expressing hVR1 show increased conductance in response to 100 nM capsaisin. The whole cell configuration of the patch clamp technique was used to record whole cell currents elicted by a voltage ramp protocol (bottom trace). The response to capsaicin was blocked by capsazepine and the effect was partially reversed after wash out of antagonist.

Figure 11 HEK293 cells stably expressing hVR1 show increased conductance in response to RTX (160 nM).

Figure 12 A, B, C. (A) HEK293 cells stably expressing hVR1 show increased conductance in response to low (pH 4.5). (B) Inhibition of the pH response by hVR1 antagonist CPZ at 1 micromolar. (C) pH response after CPZ washout.

DETAILED DESCRIPTION

The present invention relates to DNA encoding human VR1 receptor that was isolated from a human thalamus cDNA library. Human VR1 receptor, as used herein, refers to protein which can specifically function as a receptor.

The complete amino acid sequence of human VR1 receptor was not previously known, nor was the complete nucleotide sequence encoding human VR1 receptor known. This is the first reported cloning of a full length DNA molecule encoding the human VR1 receptor. It is predicted that a wide variety of cells and cell types will contain the described receptor.

Other cells and cell lines may also be suitable for use to isolate human VR1 receptor cDNA. Selection of suitable cells may be done by screening for human VR1 receptor activity in cell extracts. Human VR1 receptor activity can be monitored by performing an ^{3}H-[resiniferatoxin] binding assay (Acs et al., 1994; Szallasi and Blumberg, 1990; Szallasi et al., 1994; Szallasi et al., 1993; Szallasi et al., 1991) or by direct measurement of a capsaicin-, RTX- and/or low pH-induced Ca^{2+} influx or non-selective cation currents through the hVR1 receptor (Caterina et al., 1997). Cells that possess human VR1 receptor activity in this assay may be suitable for the isolation of human VR1 receptor DNA or mRNA.

Any of a variety of procedures known in the art may be used to molecularly clone human VR1 receptor DNA. These methods include, but are not limited to, direct functional expression of the human VR1 receptor genes following the construction of a human VR1 receptor-containing cDNA library in an appropriate expression vector system. Another method is to screen human VR1 receptor-containing cDNA library constructed in a bacteriophage or plasmid shuttle vector with a labelled oligonucleotide probe designed from the amino acid sequence of the human VR1 receptor subunits. An additional method consists of screening a human VR1 receptor-containing cDNA library constructed in a bacteriophage or plasmid

shuttle vector with a partial cDNA encoding the human VR1 receptor protein. This partial cDNA is obtained by the specific PCR amplification of human VR1 receptor DNA fragments through the design of degenerate oligonucleotide primers from the amino acid sequence of the purified human VR1 receptor protein.

Another method is to isolate RNA from human VR1 receptor-producing cells and translate the RNA into protein via an *in vitro* or an *in vivo* translation system. The translation of the RNA into a peptide a protein will result in the production of at least a portion of the human VR1 receptor protein which an be identified by, for example, immunological reactivity with an anti-human VR1 receptor antibody or by biological activity of human VR1 receptor protein. In this method, pools of RNA isolated from human VR1 receptor-producing cells can be analyzed for the presence of an RNA that encodes at least a portion of the human VR1 receptor protein. Further fractionation of the RNA pool can be done to purify the human VR1 receptor RNA from non-human VR1 receptor RNA. The peptide or protein produced by this method may be analyzed to provide amino acid sequences, which in turn are used to provide primers for production of human VR1 receptor cDNA, or the RNA used for translation can be analyzed to provide nucleotide sequences encoding human VR1 receptor and produce probes for this production of human VR1 receptor cDNA. This method is known in the art and can be found in, for example, Maniatis, T., Fritsch, E.F., Sambrook, J. in Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 1989.

It is readily apparent to those skilled in the art that other types of libraries, as well as libraries constructed from other cells or cell types, may be useful for isolating human VR1 receptor-encoding DNA. Other types of libraries include, but are not limited to, cDNA libraries derived from other cells and genomic DNA libraries that include YAC (yeast artificial chromosome) and cosmid libraries.

It is readily apparent to those skilled in the art that suitable cDNA libraries may be prepared from cells or cell lines which have human VR1 receptor activity.

The selection of cells or cell lines for use in preparing a cDNA library to isolate human VR1 receptor cDNA may be done by first measuring cell associated human VR1 receptor activity using the measurement of Capsaicin-associated biological activity or a capsaicin ligand binding assay.

Preparation of cDNA libraries can be performed by standard techniques well known in the art. Well known cDNA library construction techniques can be found for example, in Maniatis, T., Fritsch, E.F., Sambrook, J., Molecular Cloning: A Laboratory Manual, Second Edition (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989).

It is also readily apparent to those skilled in the art that DNA encoding human VR1 receptor may also be isolated from a suitable genomic DNA library. Construction of genomic DNA libraries can be performed by standard techniques well known in the art. Well known genomic DNA library construction techniques can be found in Maniatis, T., Fritsch, E.F., Sambrook, J. in Molecular Cloning: A Laboratory Manual, Second Edition (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989).

In order to clone the human VR1 receptor gene by the above methods, the amino acid sequence of human VR1 receptor may be necessary. To accomplish this, human VR1 receptor protein may be purified and partial amino acid sequence determined by automated sequencers. It is not necessary to determine the entire amino acid sequence, but the linear sequence of two regions of 6 to 8 amino acids from the protein is determined for the production of primers for PCR amplification of a partial human VR1 receptor DNA fragment.

Once suitable amino acid sequences have been identified, the DNA sequences capable of encoding them are synthesized. Because the genetic code is degenerate, more than one codon may be used to encode a particular amino acid, and therefore, the amino acid sequence can be encoded by any of a set of similar DNA oligonucleotides. Only one member of the set will be identical to the human VR1

receptor sequence but will be capable of hybridizing to human VR1 receptor DNA even in the presence of DNA oligonucleotides with mismatches. The mismatched DNA oligonucleotides may still sufficiently hybridize to the human VR1 receptor DNA to permit identification and isolation of human VR1 receptor encoding DNA. DNA isolated by these methods can be used to screen DNA libraries from a variety of cell types, from invertebrate and vertebrate sources, and to isolate homologous genes.

Purified biologically active human VR1 receptor may have several different physical forms. Human VR1 receptor may exist as a full-length nascent or unprocessed polypeptide, or as partially processed polypeptides or combinations of processed polypeptides. The full-length nascent human VR1 receptor polypeptide may be posttranslationally modified by specific proteolytic cleavage events, which result in the formation of fragments of the full-length nascent polypeptide. A fragment, or physical association of fragments may have the full biological activity associated with human VR1 receptor, however, the degree of human VR1 receptor activity may vary between individual human VR1 receptor fragments and physically associated human VR1 receptor polypeptide fragments.

The cloned human VR1 receptor DNA obtained through the methods described herein may be recombinantly expressed by molecular cloning into an expression vector containing a suitable promoter and other appropriate transcription regulatory elements, and transferred into prokaryotic or eukaryotic host cells to produce recombinant human VR1 receptor protein. Techniques for such manipulations are fully described in Maniatis, T, et al., supra, and are well known in the art.

Expression vectors are defined herein as DNA sequences that are required for the transcription of cloned copies of genes and the translation of their mRNAs in an appropriate host. Such vectors can be used to express eukaryotic genes in a variety of

hosts such as bacteria including E. coli, blue-green algae, plant cells, insect cells, fungal cells including yeast cells, and animal cells.

Specifically designed vectors allow the shuttling of DNA between hosts such as bacteria-yeast or bacteria-animal cells or bacteria-fungal cells or bacteria-invertebrate cells. An appropriately constructed expression vector should contain: an origin of replication for autonomous replication in host cells, selectable markers, a limited number of useful restriction enzyme sites, a potential for high copy number, and active promoters. A promoter is defined as a DNA sequence that directs RNA polymerase to bind to DNA and initiate RNA synthesis. A strong promoter is one that causes mRNAs to be initiated at high frequency. Expression vectors may include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses.

A variety of mammalian expression vectors may be used to express recombinant human VR1 receptor in mammalian cells. Commercially available mammalian expression vectors which may be suitable for recombinant human VR1 receptor expression, include but are not limited to, pMAMneo (Clontech), pcDNA3 (Invitrogen), pMC1neo (Stratagene), pXT1 (Stratagene), pSG5 (Stratagene), EBO-pSV2-neo (ATCC 37593) pBPV-1(8-2) (ATCC 37110), pdBPV-MMTneo(342-12) (ATCC 37224), pRSVgpt (ATCC 37199), pRSVneo (ATCC 37198), pSV2-dhfr (ATCC 37146), pUCTag (ATCC 37460), and lZD35 (ATCC 37565).

A variety of bacterial expression vectors may be used to express recombinant human VR1 receptor in bacterial cells. Commercially available bacterial expression vectors which may be suitable for recombinant human VR1 receptor expression include, but are not limited to pET vectors (Novagen) and pQE vectors (Qiagen).

A variety of fungal cell expression vectors may be used to express recombinant human VR1 receptor in fungal cells such as yeast. Commercially available fungal cell expression vectors which may be suitable for recombinant

human VR1 receptor expression include but are not limited to pYES2 (InVitrogen) and Pichia expression vector (InVitrogen).

A variety of insect cell expression vectors may be used to express recombinant human VR1 receptor in insect cells. Commercially available insect cell expression vectors that may be suitable for recombinant expression of human VR1 receptor include but are not limited to pBlueBacII (InVitrogen).

DNA encoding human VR1 receptor may be cloned into an expression vector for expression in a recombinant host cell. Recombinant host cells may be prokaryotic or eukaryotic, including but not limited to bacteria such as E. coli, fungal cells such as yeast, mammalian cells including but not limited to cell lines of human, bovine, porcine, monkey and rodent origin, and insect cells including but not limited to drosophila and silkworm derived cell lines. Cell lines derived from mammalian species which may be suitable and which are commercially available, include but are not limited to, CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), L-cells, and HEK-293 (ATCC CRL1573).

The expression vector may be introduced into host cells via any one of a number of techniques including but not limited to transformation, transfection, protoplast fusion, lipofection, and electroporation. The expression vector-containing cells are clonally propagated and individually analyzed to determine whether they produce human VR1 receptor protein. Identification of human VR1 receptor expressing host cell clones may be done by several means, including but not limited to immunological reactivity with anti-human VR1 receptor antibodies, and the presence of host cell-associated human VR1 receptor activity.

Expression of human VR1 receptor DNA may also be performed using *in vitro* produced synthetic mRNA. Synthetic mRNA or mRNA isolated from human VR1 receptor producing cells can be efficiently translated in various cell-free

systems, including but not limited to wheat germ extracts and reticulocyte extracts, as well as efficiently translated in cell based systems, including but not limited to microinjection into frog oocytes, with microinjection into frog oocytes being generally preferred.

To determine the human VR1 receptor DNA sequence(s) that yields optimal levels of human VR1 receptor activity and/or human VR1 receptor protein, human VR1 receptor DNA molecules including, but not limited to, the following can be constructed: the full-length open reading frame of the human VR1 receptor cDNA encoding the approximately 95,048 kDa protein from approximately base 1 to approximately base 2517 (these numbers correspond to first nucleotide of first methionine and last nucleotide before the first stop codon) and several constructs containing portions of the cDNA encoding human VR1 receptor protein. All constructs can be designed to contain none, all or portions of the 5' or the 3' untranslated region of human VR1 receptor cDNA. Human VR1 receptor activity and levels of protein expression can be determined following the introduction, both singly and in combination, of these constructs into appropriate host cells. Following determination of the human VR1 receptor DNA cassette yielding optimal expression in transient assays, this human VR1 receptor DNA construct is transferred to a variety of expression vectors, for expression in host cells including, but not limited to, mammalian cells, baculovirus-infected insect cells, E. coli, and the yeast S. cerevisiae.

Host cell transfectants and microinjected oocytes may be used to assay both the levels of human VR1 receptor channel activity and levels of human VR1 receptor protein by the following methods. In the case of recombinant host cells, this involves the co-transfection of one or possibly two or more plasmids, containing the human VR1 receptor DNA encoding one or more fragments or subunits. In the case of oocytes, this involves the co-injection of synthetic RNAs for human VR1 receptor protein. Following an appropriate period of time to allow for expression, cellular

protein is metabolically labelled with, for example ^{35}S-methionine for 24 hours, after which cell lysates and cell culture supernatants are harvested and subjected to immunoprecipitation with polyclonal antibodies directed against the human VR1 receptor protein.

Other methods for detecting human VR1 receptor activity involve the direct measurement of human VR1 receptor activity in whole cells transfected with human VR1 receptor cDNA or oocytes injected with human VR1 receptor mRNA. Human VR1 receptor activity is measured by specific ligand binding and biological characteristics of the host cells expressing human VR1 receptor DNA. In the case of recombinant host cells expressing human VR1 receptor patch voltage clamp techniques can be used to measure receptor activity and quantitate human VR1 receptor protein. In the case of oocytes patch clamp as well as two-electrode voltage clamp techniques can be used to measure VR1 receptor activity and quantitate human VR1 receptor protein by determining single channel and whole cell conductances.

Levels of human VR1 receptor protein in host cells are quantitated by immunoaffinity and/or ligand affinity techniques. Cells expressing human VR1 receptor can be assayed for the number of human VR1 receptor molecules expressed by measuring the amount of radioactive ligand binding to cell membranes. Human VR1 receptor-specific affinity beads or human VR1 receptor-specific antibodies are used to isolate for example ^{35}S-methionine labelled or unlabelled human VR1 receptor protein. Labelled human VR1 receptor protein is analyzed by SDS-PAGE. Unlabelled human VR1 receptor protein is detected by Western blotting, ELISA or RIA assays employing human VR1 receptor specific antibodies.

Because the genetic code is degenerate, more than one codon may be used to encode a particular amino acid, and therefore, the amino acid sequence can be encoded by any of a set of similar DNA oligonucleotides. Only one member of the set will be identical to the human VR1 receptor sequence but will be capable of hybridizing to human VR1 receptor DNA even in the presence of DNA

oligonucleotides with mismatches under appropriate conditions. Under alternate conditions, the mismatched DNA oligonucleotides may still hybridize to the human VR1 receptor DNA to permit identification and isolation of human VR1 receptor encoding DNA.

DNA encoding human VR1 receptor from a particular organism may be used to isolate and purify homologues of human VR1 receptor from other organisms. To accomplish this, the first human VR1 receptor DNA may be mixed with a sample containing DNA encoding homologues of human VR1 receptor under appropriate hybridization conditions. The hybridized DNA complex may be isolated and the DNA encoding the homologous DNA may be purified therefrom.

It is known that there is a substantial amount of redundancy in the various codons that code for specific amino acids. Therefore, this invention is also directed to those DNA sequences that contain alternative codons that code for the eventual translation of the identical amino acid. For purposes of this specification, a sequence bearing one or more replaced codons will be defined as a degenerate variation. Also included within the scope of this invention are mutations either in the DNA sequence or the translated protein which do not substantially alter the ultimate physical properties of the expressed protein. For example, substitution of valine for leucine, arginine for lysine, or asparagine for glutamine may not cause a change in functionality of the polypeptide.

It is known that DNA sequences coding for a peptide may be altered so as to code for a peptide having properties that are different than those of the naturally occurring peptide. Methods of altering the DNA sequences include, but are not limited to site directed mutagenesis. Examples of altered properties include but are not limited to changes in the affinity of an enzyme for a substrate or a receptor for a ligand.

As used herein, a "functional derivative" of human VR1 receptor is a compound that possesses a biological activity (either functional or structural) that is

substantially similar to the biological activity of human VR1 receptor. The term "functional derivatives" is intended to include the "fragments," "variants," "degenerate variants," "analogs" and "homologues" or to "chemical derivatives" of human VR1 receptor. The term "fragment" is meant to refer to any polypeptide subset of human VR1 receptor. The term "variant" is meant to refer to a molecule substantially similar in structure and function to either the entire human VR1 receptor molecule or to a fragment thereof. A molecule is "substantially similar" to human VR1 receptor if both molecules have substantially similar structures or if both molecules possess similar biological activity. Therefore, if the two molecules possess substantially similar activity, they are considered to be variants even if the structure of one of the molecules is not found in the other or even if the two amino acid sequences are not identical. The term "analog" refers to a molecule substantially similar in function to either the entire human VR1 receptor molecule or to a fragment thereof.

Following expression of human VR1 receptor in a recombinant host cell, human VR1 receptor protein may be recovered to provide human VR1 receptor in active form. Several human VR1 receptor purification procedures are available and suitable for use. As described above for purification of human VR1 receptor from natural sources, recombinant human VR1 receptor may be purified from cell lysates and extracts, or from conditioned culture medium, by various combinations of, or individual application of salt fractionation, ion exchange chromatography, size exclusion chromatography, hydroxylapatite adsorption chromatography and hydrophobic interaction chromatography.

In addition, recombinant human VR1 receptor can be separated from other cellular proteins by use of an immunoaffinity column made with monoclonal or polyclonal antibodies specific for full length nascent human VR1 receptor, polypeptide fragments of human VR1 receptor or human VR1 receptor subunits.

Monospecific antibodies to human VR1 receptor are purified from mammalian antisera containing antibodies reactive against human VR1 receptor or are prepared as monoclonal antibodies reactive with human VR1 receptor using the technique of Kohler and Milstein, *Nature* 256: 495-497 (1975). Monospecific antibody as used herein is defined as a single antibody species or multiple antibody species with homogenous binding characteristics for human VR1 receptor. Homogenous binding as used herein refers to the ability of the antibody species to bind to a specific antigen or epitope, such as those associated with the human VR1 receptor, as described above. Human VR1 receptor specific antibodies are raised by immunizing animals such as mice, rats, guinea pigs, rabbits, goats, horses and the like, with rabbits being preferred, with an appropriate concentration of human VR1 receptor either with or without an immune adjuvant.

Preimmune serum is collected prior to the first immunization. Each animal receives between about 0.1 mg and about 1000 mg of human VR1 receptor associated with an acceptable immune adjuvant. Such acceptable adjuvants include, but are not limited to, Freund's complete, Freund's incomplete, alum-precipitate, water in oil emulsion containing Corynebacterium parvum and tRNA. The initial immunization consists of human VR1 receptor in, preferably, Freund's complete adjuvant at multiple sites either subcutaneously (SC), intraperitoneally (IP) or both. Each animal is bled at regular intervals, preferably weekly, to determine antibody titer. The animals may or may not receive booster injections following the initial immunization. Those animals receiving booster injections are generally given an equal amount of the antigen in Freund's incomplete adjuvant by the same route. Booster injections are given at about three-week intervals until maximal titers are obtained. At about 7 days after each booster immunization or about weekly after a single immunization, the animals are bled, the serum collected, and aliquots are stored at about -20°C.

Monoclonal antibodies (mAb) reactive with human VR1 receptor are prepared by immunizing inbred mice, preferably Balb/c, with human VR1 receptor. The mice

are immunized by the IP or SC route with about 0.1 mg to about 10 mg, preferably about 1 mg, of human VR1 receptor in about 0.5 ml buffer or saline incorporated in an equal volume of an acceptable adjuvant, as discussed above. Freund's complete adjuvant is preferred. The mice receive an initial immunization on day 0 and are rested for about 3 to about 30 weeks. Immunized mice are given one or more booster immunizations of about 0.1 to about 10 mg of human VR1 receptor in a buffer solution such as phosphate buffered saline by the intravenous (IV) route. Lymphocytes, from antibody positive mice, preferably splenic lymphocytes, are obtained by removing spleens from immunized mice by standard procedures known in the art. Hybridoma cells are produced by mixing the splenic lymphocytes with an appropriate fusion partner, preferably myeloma cells, under conditions that will allow the formation of stable hybridomas. Fusion partners may include, but are not limited to: mouse myelomas P3/NS1/Ag 4-1; MPC-11; S-194 and Sp 2/0, with Sp 2/0 being generally preferred. The antibody producing cells and myeloma cells are fused in polyethylene glycol, about 1000 mol. wt., at concentrations from about 30% to about 50%. Fused hybridoma cells are selected by growth in hypoxanthine, thymidine and aminopterin supplemented Dulbecco's Modified Eagles Medium (DMEM) by procedures known in the art. Supernatant fluids are collected from growth positive wells on about days 14, 18, and 21 and are screened for antibody production by an immunoassay such as solid phase immunoradioassay (SPIRA) using human VR1 receptor as the antigen. The culture fluids are also tested in the Ouchterlony precipitation assay to determine the isotype of the mAb. Hybridoma cells from antibody positive wells are cloned by a technique such as the soft agar technique of MacPherson, Soft Agar Techniques, in Tissue Culture Methods and Applications, Kruse and Paterson, Eds., Academic Press, 1973.

Monoclonal antibodies are produced *in vivo* by injection of pristane primed Balb/c mice, approximately 0.5 ml per mouse, with about 2 x 10^6 to about 6 x 10^6 hybridoma cells about 4 days after priming. Ascites fluid is collected at

approximately 8-12 days after cell transfer and the monoclonal antibodies are purified by techniques known in the art.

In vitro production of anti-human VR1 receptor mAb is carried out by growing the hybridoma in DMEM containing about 2% fetal calf serum to obtain sufficient quantities of the specific mAb. The mAb are purified by techniques known in the art.

Antibody titers of ascites or hybridoma culture fluids are determined by various serological or immunological assays which include, but are not limited to, precipitation, passive agglutination, enzyme-linked immunosorbent antibody (ELISA) technique and radioimmunoassay (RIA) techniques. Similar assays are used to detect the presence of human VR1 receptor in body fluids or tissue and cell extracts.

It is readily apparent to those skilled in the art that the above described methods for producing monospecific antibodies may be utilized to produce antibodies specific for human VR1 receptor polypeptide fragments, or full-length nascent human VR1 receptor polypeptide, or the individual human VR1 receptor subunits. Specifically, it is readily apparent to those skilled in the art that monospecific antibodies may be generated which are specific for only one human VR1 receptor subunit or the fully functional receptor.

Human VR1 receptor antibody affinity columns are made by adding the antibodies to Affigel-10 (Bio-Rad), a gel support which is activated with N-hydroxysuccinimide esters such that the antibodies form covalent linkages with the agarose gel bead support. The antibodies are then coupled to the gel via amide bonds with the spacer arm. The remaining activated esters are then quenched with 1M ethanolamine HCl (pH 8). The column is washed with water followed by 0.23 M glycine HCl (pH 2.6) to remove any non-conjugated antibody or extraneous protein. The column is then equilibrated in phosphate buffered saline (pH 7.3) and the cell culture supernatants or cell extracts containing human VR1 receptor or human VR1

receptor subunits are slowly passed through the column. The column is then washed with phosphate buffered saline until the optical density (A_{280}) falls to background, then the protein is eluted with 0.23 M glycine-HCl (pH 2.6). The purified human VR1 receptor protein is then dialyzed against phosphate buffered saline.

DNA clones, termed human VR1 receptor, are identified which encode proteins that, when expressed in a recombinant host cell, form receptors sensitive to capsaicin. The expression of human VR1 receptor DNA results in the reconstitution of the properties observed in oocytes injected with human VR1 receptor-encoding poly $(A)^+$ RNA, including direct activation with the appropriate ligands.

The present invention is also directed to methods for screening for compounds that modulate the expression of DNA or RNA encoding human VR1 receptor as well as the function of human VR1 receptor protein *in vivo*. Compounds that modulate these activities may be DNA, RNA, peptides, proteins, or non-proteinaceous organic molecules. Compounds may modulate by increasing or attenuating the expression of DNA or RNA encoding human VR1 receptor, or the function of human VR1 receptor protein. Compounds that modulate the expression of DNA or RNA encoding human VR1 receptor or the function of human VR1 receptor protein may be detected by a variety of assays. The assay may be a simple "yes/no" assay to determine whether there is a change in expression or function. The assay may be made quantitative by comparing the expression or function of a test sample with the levels of expression or function in a standard sample. Modulators identified in this process are useful as therapeutic agents.

Kits containing human VR1 receptor DNA or RNA, antibodies to human VR1 receptor, or human VR1 receptor protein may be prepared. Such kits are used to detect DNA that hybridizes to human VR1 receptor DNA or to detect the presence of human VR1 receptor protein or peptide fragments in a sample. Such characterization is useful for a variety of purposes including but not limited to forensic analyses, diagnostic applications, and epidemiological studies.

The DNA molecules, RNA molecules, recombinant protein and antibodies of the present invention may be used to screen and measure levels of human VR1 receptor DNA, human VR1 receptor RNA or human VR1 receptor protein. The recombinant proteins, DNA molecules, RNA molecules and antibodies lend themselves to the formulation of kits suitable for the detection and typing of human VR1 receptor. Such a kit would comprise a compartmentalized carrier suitable to hold in close confinement at least one container. The carrier would further comprise reagents such as recombinant human VR1 receptor protein or anti-human VR1 receptor antibodies suitable for detecting human VR1 receptor. The carrier may also contain a means for detection such as labeled antigen or enzyme substrates or the like.

Nucleotide sequences that are complementary to the human VR1 receptor encoding DNA sequence can be synthesized for antisense therapy. These antisense molecules may be DNA, stable derivatives of DNA such as phosphorothioates or methylphosphonates, RNA, stable derivatives of RNA such as 2'-*O*-alkylRNA, or other human VR1 receptor antisense oligonucleotide mimetics. Human VR1 receptor antisense molecules may be introduced into cells by microinjection, liposome encapsulation or by expression from vectors harboring the antisense sequence. Human VR1 receptor antisense therapy may be particularly useful for the treatment of diseases where it is beneficial to reduce human VR1 receptor activity.

Human VR1 receptor gene therapy may be used to introduce human VR1 receptor into the cells of target organisms. The human VR1 receptor gene can be ligated into viral vectors that mediate transfer of the human VR1 receptor DNA by infection of recipient host cells. Suitable viral vectors include retrovirus, adenovirus, adeno-associated virus, herpes virus, vaccinia virus, polio virus and the like. Alternatively, human VR1 receptor DNA can be transferred into cells for gene therapy by non-viral techniques including receptor-mediated targeted DNA transfer using ligand-DNA conjugates or adenovirus-ligand-DNA conjugates, lipofection

membrane fusion or direct microinjection. These procedures and variations thereof are suitable for *ex vivo* as well as *in vivo* human VR1 receptor gene therapy. Human VR1 receptor gene therapy may be particularly useful for the treatment of diseases where it is beneficial to elevate human VR1 receptor activity.

Pharmaceutically useful compositions comprising human VR1 receptor DNA, human VR1 receptor RNA, or human VR1 receptor protein, or modulators of human VR1 receptor activity, may be formulated according to known methods such as by the admixture of a pharmaceutically acceptable carrier. Examples of such carriers and methods of formulation may be found in Remington's Pharmaceutical Sciences. To form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the protein, DNA, RNA, or modulator.

Therapeutic or diagnostic compositions of the invention are administered to an individual in amounts sufficient to treat or diagnose disorders in which modulation of human VR1 receptor-related activity is indicated. The effective amount may vary according to a variety of factors such as the individual's condition, weight, sex and age. Other factors include the mode of administration. The pharmaceutical compositions may be provided to the individual by a variety of routes such as subcutaneous, topical, oral and intramuscular.

The term "chemical derivative" describes a molecule that contains additional chemical moieties that are not normally a part of the base molecule. Such moieties may improve the solubility, half-life, absorption, etc. of the base molecule. Alternatively the moieties may attenuate undesirable side effects of the base molecule or decrease the toxicity of the base molecule. Examples of such moieties are described in a variety of texts, such as Remington's Pharmaceutical Sciences.

Compounds identified according to the methods disclosed herein may be used alone at appropriate dosages defined by routine testing in order to obtain optimal inhibition of the human VR1 receptor or its activity while minimizing any potential

toxicity. In addition, co-administration or sequential administration of other agents may be desirable.

The present invention also has the objective of providing suitable topical, oral, systemic and parenteral pharmaceutical formulations for use in the novel methods of treatment of the present invention. The compositions containing compounds or modulators identified according to this invention as the active ingredient for use in the modulation of human VR1 receptor receptors can be administered in a wide variety of therapeutic dosage forms in conventional vehicles for administration. For example, the compounds or modulators can be administered in such oral dosage